PERGAMON

European Journal of Cancer 39 (2003) 829–837

European Journal of Cancer

www.ejconline.com

Study of *in vitro* and *in vivo* effects of the piperidine nitroxide Tempol—a potential new therapeutic agent for gliomas

M.B. Gariboldi[a], R. Ravizza[a], C. Petterino[c], M. Castagnaro[c], G. Finocchiaro[b], E. Monti[a,*]

[a]*DBSF, Laboratory of Pharmacology, University of Insubria, Via A. da Giussano, 12, 21052 Busto Arsizio (VA), Italy*
[b]*Istituto Neurologico Besta, Via Celoria 20129 Milan, Italy*
[c]*Department of Public Health, Comparative Pathology and Veterinary Hygiene, Strada Romea 16, 35020 Agripolis (PD), Italy*

Received 3 April 2002; received in revised form 19 August 2002; accepted 23 October 2002

Abstract

The identification of novel therapeutic agents for the management of malignant gliomas represents an area of active research. Here, we show that Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; TPL), a stable nitroxide free radical, inhibits the growth of C6 glioma cells both *in vitro* and *in vivo*. Morphological features of apoptosis were apparent in C6 cells following *in vitro* treatment with TPL. Cell death was preceded by dose-dependent increase in $p21^{waf1/cip1}$ expression, without apparent stabilisation of the *TP53* gene product. When C6 cells were grown as xenografts in nude mice, treatment with TPL induced a significant dose-dependent decrease in tumour growth, without signs of general or organ toxicity. Tumours from treated mice showed an increase in the number of apoptotic cells and a decrease in the rate of neo-vascularisation compared with tumours from control mice. Our findings suggest a potential use for TPL as a novel antiproliferative agent for the treatment of malignant gliomas.

Keywords: Glioma; Tempol; Antitumour effects; $p21^{waf1/cip1}$

1. Introduction

Malignant gliomas are the most common primary tumours of the adult central nervous system (CNS) [1]. Current therapies include surgical removal and irradiation. However, the infiltrative behaviour of malignant gliomas often precludes their complete eradication, and even after irradiation the prognosis of high-grade glioma patients remains poor. Moreover, chemotherapeutic treatments for gliomas are often ineffective because of the intrinsic chemoresistance of these tumours [2]. The identification of novel therapeutic agents able to inhibit the growth of this tumour type is therefore essential to improve the prognosis of glioma patients and is currently an area of active research.

The aim of the present study was to investigate the activity of the piperidine nitroxide Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; TPL; Fig. 1) against malignant glioma cells, *in vitro* as well as in an *in vivo* xenograft model. A number of observations have suggested that TPL, a stable free radical, might be a useful addition to the scant repertoire of drugs currently employed in the treatment of gliomas. First, TPL showed antiproliferative activity against a panel of tumour cell lines, with some degree of selectivity between neoplastic and non-neoplastic cells of similar lineage [3]. Second, TPL can induce an increase in *waf1/cip1* expression in human leukaemic HL-60 cells, in spite of the complete lack of the *TP53* gene product in this cell line [4], and the protein product of the *waf1/cip1* gene, the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$, has been demonstrated to increase the susceptibility of at least two different human glioma cell lines to apoptosis induced by cytotoxic agents [5]. Third, TPL is a low molecular weight membrane-permeable molecule that can easily reach the CNS [6]. Fourth, TPL has been shown to act as a radiosensitiser on cells subjected to hypoxic conditions [7]. As gliomas typically develop hypoxic regions, which possibly account for the poor

* Corresponding author. Tel.: +39-0331-339415; fax: +39-0331-339459.
E-mail address: elena.monti@uninsubria.it (E. Monti).

0959-8049/03/$ - see front matter
PII: S0959-8049(02)00742-6

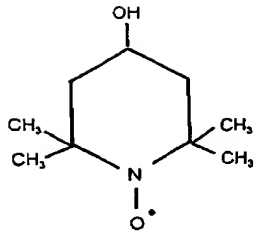


Fig. 1. Structural formula of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol, TPL).

response of these tumours to radiation therapy, treatment with TPL might improve the effectiveness of this therapeutic strategy.

The present study was performed on C6 rat glioma cells, grown both *in vitro* as monolayers and *in vivo* as xenografts in nude mice.

2. Materials and methods

2.1. Reagents

4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol, TPL), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and standard reagents were purchased from Sigma-Aldrich s.r.l. (Milan, Italy).

2.2. Cells and in vitro culture conditions

C6 cells, a rat glioma cell line, were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Mascia-Brunelli, Milan, Italy) supplemented with 10% fetal bovine serum (FBS) (Mascia Brunelli) at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Cytotoxicity assays and effects on cell growth

The antiproliferative effect of TPL was assessed by the MTT assay [8]. Briefly, 10^4 cells/ml were seeded onto 96-well plates and allowed to grow for 24 h prior to treatment with different concentrations of TPL (0.05–10.0 mM). Three different exposure schedules were used: (a) 24 h, (b) 24 h followed by 72 h in TPL-free medium; and (c) 96 h continuous exposure. At the end of the appointed time periods, MTT was added to each well (final concentration 0.4 mg/ml); formazan crystals formed through MTT metabolism by viable cells were dissolved in dimethylsulphoxide (DMSO) and optical densities were measured at 570 nm using a Universal Microplate Reader EL800 (Bio-Tek Instruments).

2.4. Visualisation of apoptotic cells

Apoptotic cells were visualised by fluorescence microscopy. C6 cells were seeded onto coverslips, allowed to grow for 24 h and subsequently treated with TPL for up to 96 h; untreated cells were grown as controls for the same periods of time. At different times, detached cells were collected, washed in phosphate-buffered saline (PBS) and fixed in 70% methanol for at least 30 min at −20 °C; methanol was removed by centrifugation and cells were resuspended in PBS containing RNAse A (1 KU/ml), propidium iodide (50 μg/ml) and 0.05% Nonidet (N)P40. After 30 min on ice, cells were mounted onto glass slides with Mowiol 4-88 and observed with a Zeiss Axiophot photomicroscope equipped for epifluorescence. Cells still attached to coverslips were also fixed in 70% methanol, washed in PBS and incubated as described for the detached cells. Cells (attached and detached) were also observed by fluorescence microscopy after performing the TdT-mediated dUTP nick-end-labelling (TUNEL) reaction using the *In Situ Cell Death Detection Kit—Fluorescein* (Roche) according to the manufacturer's instructions.

2.5. Preparation of cell extracts and immunoblotting

Western blot analysis was carried out to detect the expression of $p21^{waf1/cip1}$ and p53 in protein extracts from control and TPL-treated samples. Cells were harvested following 24-h exposure to TPL (1.0, 2.5 or 5.0 mM) and resuspended in lysis buffer (1% (v/v) NP40, leupeptin 10 μg/ml and aprotinin 10 μg/ml in PBS). Protein concentration was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). 10 μg of protein extract/lane were loaded onto polyacrylamide gels and separated under denaturing conditions. Protein samples were then transferred onto Immobilon P membranes (Millipore, Bedford, MA, USA) and Western blot analysis was performed using standard techniques and a mouse monoclonal antibody (Pab24; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), that recognises rat and murine forms of p53, and a rabbit polyclonal antibody raised against mouse, rat and human $p21^{waf1/cip1}$ (Santa Cruz Biotechnology, Inc). Western blots were also performed using an anti mouse-α-tubulin antibody (Santa Cruz Biotechnology, Inc), to verify equal loading of the samples. All antibodies were diluted according to the manufacturer's instructions. Proteins were visualised using a peroxidase-conjugated anti-mouse/rabbit secondary antibody and the BM Chemiluminescence Western Blot Kit (Boehringer Mannheim, Mannheim, Germany). Densitometric analysis of immunoreactive bands was performed using the Image Master VDH (Pharmacia Biotech).

2.6. In vivo *treatments*

Procedures involving animals conformed with institutional guidelines that comply with national and international laws and policies [9]. During the *in vivo* experiments, animals in all of the experimental groups were examined daily for decreased physical activity or other signs of disease.

A 14-day endpoint was set, due to the rapid growth kinetics of the implanted tumour cells, in order to keep the distress and discomfort of the experimental animals to a minimum.

C6 cells (3×10^6 cells/ml in 0.9% NaCl) were injected subcutaneously (s.c.) into 40-day athymic female nude mice. As soon as tumours became palpable, approximately 4 days after the injection, an osmotic pump (Alzet) releasing 0.5 μl/h of TPL solution (or saline) for 14 days was implanted s.c. into each mouse. Three groups of mice were included in each experiment, the first (control) receiving saline only, the second (TPL A) receiving 0.25 g/ml of TPL in sterile saline through the osmotic pump and the third (TPL B) receiving 0.375 g/ml of TPL in sterile saline through the osmotic pump, plus two daily intraperitoneal (i.p.) injections of the nitroxide (100 mg/kg), 5 days/week.

Variations in body weight and tumour size were recorded throughout the 14-day treatment period, and animals were examined for signs of general toxicity. Tumour size was evaluated by measuring the two major diameters (length and width) of the tumours with callipers and by calculating tumour volumes according to the following formula: $(\text{length}\times\text{width}^2)/2$. Differences in tumour growth and body weight gain throughout the experimental period were evaluated by an ANOVA design for repeated measures; differences at specific time points were analysed by one-way ANOVA. At the end of the treatment, the animals were euthanised using a pentobarbital overdose and subsequent cervical dislocation and the tumours were removed and fixed in 4% *p*-formaldehyde for subsequent immunohistochemical analyses. Necroscopic examination was performed on all the animals, to identify any signs of organ toxicity.

2.7. *Immunohistochemical staining and evaluation*

Formalin-fixed and paraffin-embedded sections were cut into 4-μm sections for microscopic examination, and 3-μm sections for immunohistochemistry and routine examination, and subsequently stained with haematoxylin and eosin. Immunohistochemical staining for von Willebrand Factor (vWF) was performed using the EnVision method. Briefly, tissue sections were deparaffinised, rehydrated in water, incubated in a 1:4 solution of 3% hydrogen peroxide and methanol to block endogenous peroxidase and washed with PBS. The sections were placed in plastic coplin jars containing 10% sodium citrate, heated at 120 °C for 2 min and allowed to cool down for 15 min, rinsed in PBS and incubated with 5% bovine serum albumin (BSA) in PBS at room temperature, to block any non-specific antibody reactions. Sections were then incubated at room temperature with the polyclonal anti-vWF antibody (A0082 DAKO, A/S Denmark; dilution 1:400) for 1 h, rinsed in PBS, incubated at room temperature with EnVision + TM Peroxidase anti-rabbit (DAKO Corporation, Carpinteria CA, USA), and stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB SK-4100, Vector Laboratories, Inc., Burlingame, CA, USA) as a chromogen. Sections were counterstained with Harris haematoxylin. Negative controls were prepared using normal mouse rabbit IgG as the primary antibody and resulted in no dectectable staining.

The extent of tumour vascularisation was evaluated by averaging microvessel counts/40× field performed on a total of 10 fields/tumour sample [10].

Apoptosis was evaluated using the TUNEL-based ApopTag® Plus Peroxidase *in situ* apoptosis detection kit (s7101, Intergen Company, Purchase, NY, USA). The extent of apoptotic damage was evaluated semi-quantitatively according to the following scoring system, based on the percent area staining positively for the TUNEL reaction in each preparation:

0	no staining
1	0–25%
2	26–50%
3	51–75%
5	76–100%

Data were evaluated by means of the Wilcoxon's non-parametric test.

3. Results

3.1. *In vitro effects of TPL*

Fig. 2 shows the dose–response curves obtained for C6 cells after exposure to TPL for 24 h, 24 h plus 72 h in TPL-free medium and 96 h continuous exposure. The IC_{50} values calculated from these cytotoxicity data were 6.45 ± 0.25, 0.94 ± 0.07 and 0.198 ± 0.04 mM (mean ± standard error of the mean (S.E.M) respectively, indicating that continuous exposure is required to effectively inhibit C6 cell growth.

3.2. *Visualisation of apoptotic cells*

Fig. 3 shows the effects of 72 h exposure to 1 mM TPL in C6 cells following incubation with propidium

iodide. Attached cells were dramatically reduced in number in the TPL-treated preparations (Fig. 3c) compared with the untreated cells (Fig. 3a), but only a low percentage of cells displayed morphologically altered nuclei. Detached cells were far more abundant after TPL treatment and propidium iodide staining indicated the presence of myriads of particles, corresponding to nuclear/cellular fragments of variable sizes (Fig. 3d). In contrast, the few cells in the supernatant collected from untreated cell preparations exhibited uniformly large rounded nuclei (Fig. 3b). The observed effects are time-dependent, as a progressive detachment of cells, with a parallel increase in the number of fragments in the supernatants was observed over time (24–96 h). These observations were confirmed by data obtained on pooled attached and detached cells following the TUNEL reaction (Table 1): TPL-treated cells exhibited a significantly higher percentage of apoptotic cells than controls; however, no significant differences were observed in the percentage of apoptotic cells after 24 and 72 h; i.e. the amount of apoptosis did not increase over time 60% (24 h) versus 67% (72 h).

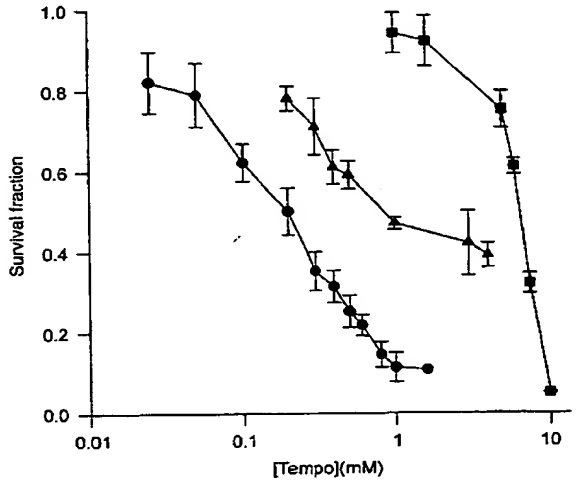


Fig. 2. Dose–response curves of C6 cells after exposure to TPL for 24 h (■), 24 h + 72 h in TPL-free medium (▲) and 96 h (●).

Table 1
Percentage of apoptotic cells following TPL treatment

	24 h	72 h
Controls	4±2.0%	7±2.8%
TPL 1.0 mM	60±12.3%*	67±15%*

TPL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl. Pooled attached and detached cells were subjected to the TUNEL reaction and analysed by fluorescence microscopy. Three 50-cell fields/sample were counted for fluorescein-positive cells. *$P < 0.01$ versus controls.

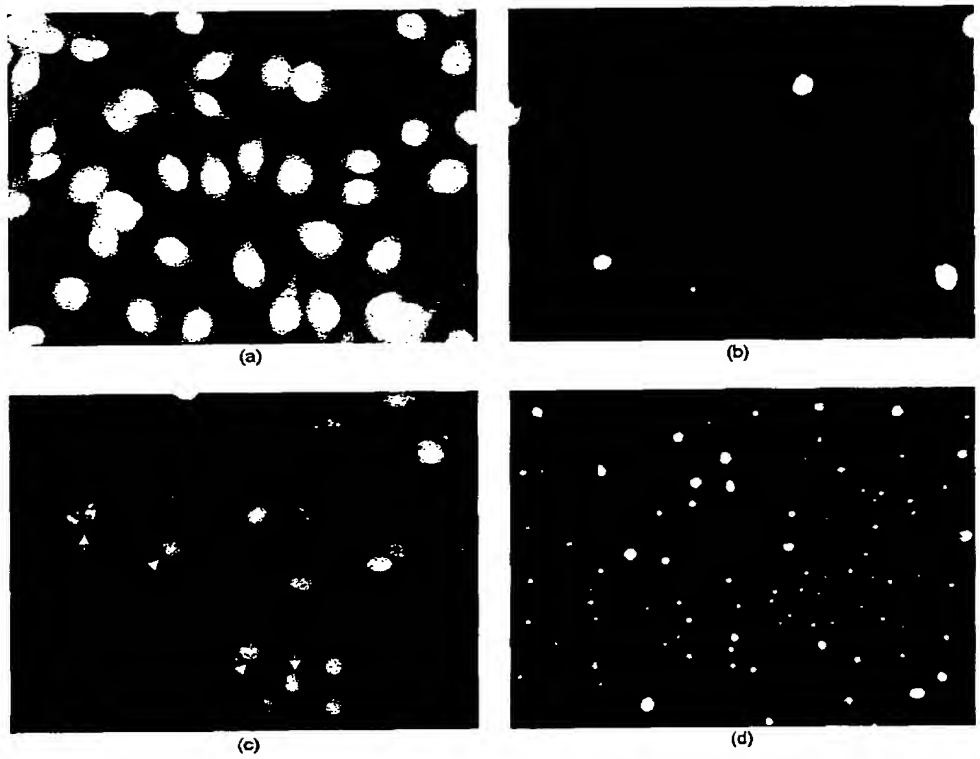


Fig. 3. Visualisation of apoptotic nuclei by fluorescent microscopy in attached and detached C6 cells following exposure to 1 mM TPL for 72 h: (a) control, attached cells; (b) control, supernatant; (c) TPL-treated, attached cells; (d) TPL-treated, supernatant.

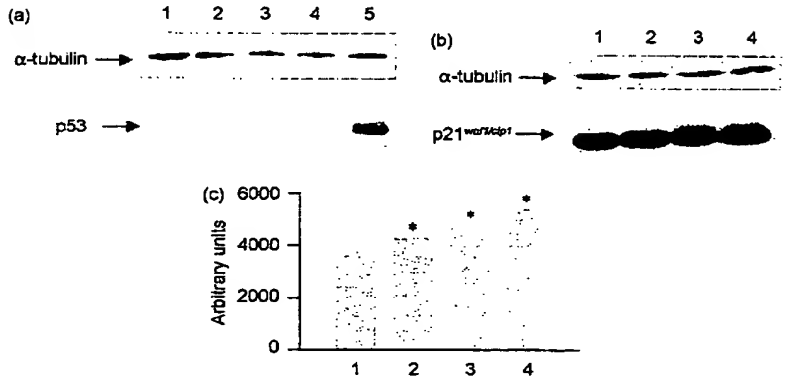


Fig. 4. Western blot analysis of p53 (a) and $p21^{waf1/cip1}$ (b) in C6 cells following TPL treatment for 24 h (lane 1: control, lane 2: TPL 1.0 mM, lane 3: TPL 2.5 mM, lane 4: TPL 5.0 mM, lane 5: p53 positive control). Densitometric analysis of $p21^{waf1/cip1}$ bands (c) (1: control; 2: TPL 1.0 mM; 3: TPL 2.5 mM, 4: TPL 5 mM. $^*P<0.05$ versus control).

3.3. Immunodetection of p53 and $p21^{waf1/cip1}$ protein levels

Western blot analysis with an anti-p53 antibody indicated that C6 cells do not express detectable levels of p53 protein, and 24-h treatment with TPL 1.0, 2.5 and 5.0 mM did not induce p53 expression (Fig. 4a). In contrast, an intense $p21^{waf1/cip1}$ immunoreactive band was detected even in the untreated cells, and its intensity increased in a concentration-dependent manner following 24 h exposure to TPL (Fig. 4b and c).

3.4. In vivo effects of TPL

The time course of C6 xenograft growth in control and TPL-treated nude mice is shown in Fig. 5. Significant inhibition of tumour growth was observed in animals treated with TPL according to both schedules, with a more potent effect in the group receiving the higher dose. Animals in both TPL-treated groups displayed a slight (non-significant) decrease in body weight compared with controls (Fig. 6). No signs of general or organ-directed toxicity were detected during TPL treatment or upon necroscopy at the end of the treatment period.

4. vWF expression and evaluation of apoptosis in the C6 xenografts

Table 2 shows the mean values ±S.E.M. for vessels counts/40× field, calculated on a total of 10 fields/sample. A decrease in microvessel counts can be observed for tumours from mice treated with the higher TPL concentration. Fig. 7 shows tumour sections (100×) from control and TPL-treated (high-dose group) mice at the end of the 2-week treatment period. The control tumours (a) showed several organised microvessels, whereas the TPL-treated tumours (b) displayed a lower

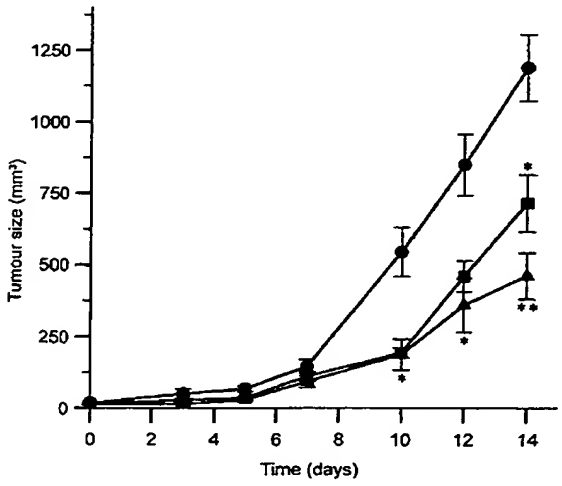


Fig. 5. Time course of C6 xenograft growth in control (●) and treated (■ TPL 0.25 g/ml; ◆ TPL 0.375 g/ml + intraperitoneally (i.p.), nitroxide 100 mg/kg twice daily, 5 days/week) mice. $^*P<0.05$ versus controls; $^{**}P<0.05$ versus controls and TPL 0.25 g/ml.

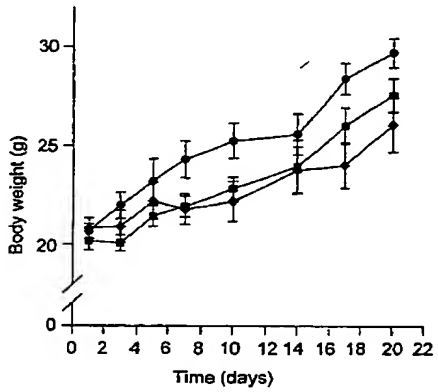
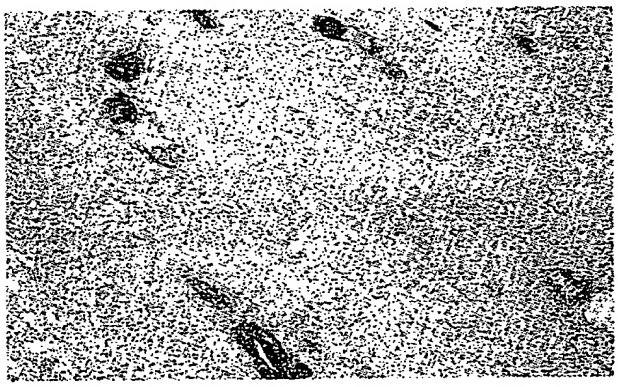


Fig. 6. Time course of body weight in control (●) and treated (■ TPL 0.25 g/ml; ◆ TPL 0.375 g/ml + i.p., nitroxide 100 mg/kg twice daily, 5 days/week) mice.

Table 2
Microvessel densities in C6 xenografts excised from control mice and mice treated with TPL according to the two different schedules.

	Microvessel density
Control	3.4±0.33
TPL (schedule A)[a]	3.44±0.09
TPL (schedule B)[a]	2.45±0.24[b]

(Mean ± standard error of the mean (S.E.M.) for cell counts/40× field, calculated on a total of 10 fields/sample.
[a] See text for a description of the two treatment schedules.
[b] $P<0.05$ versus control.


(a)

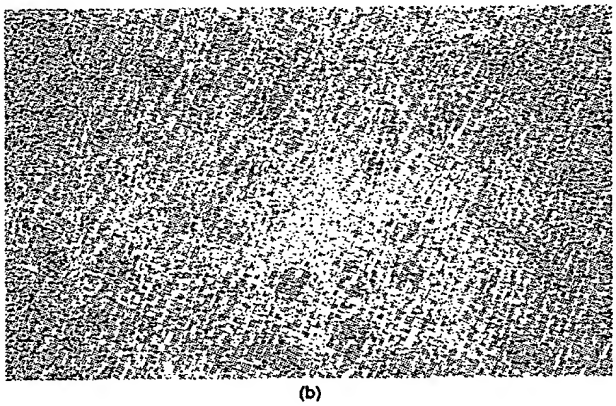
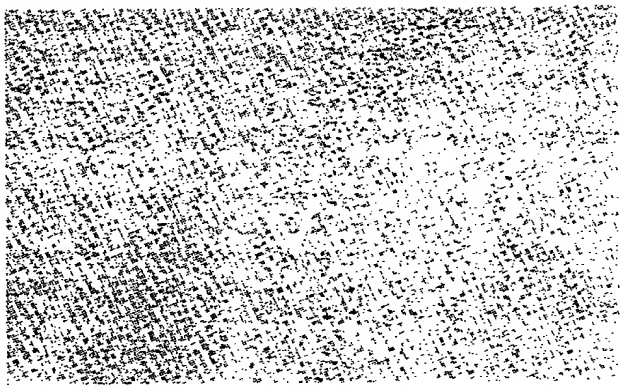

(b)

Fig. 7. Visualisation of microvessel density in C6 xenograft after treatment with TPL (magnification 100×): (a) tumour from control mice; (b) tumour from TPL 0.375 g/ml+i.p. (nitroxide 100 mg/kg twice daily, 5 days/week) treated mice.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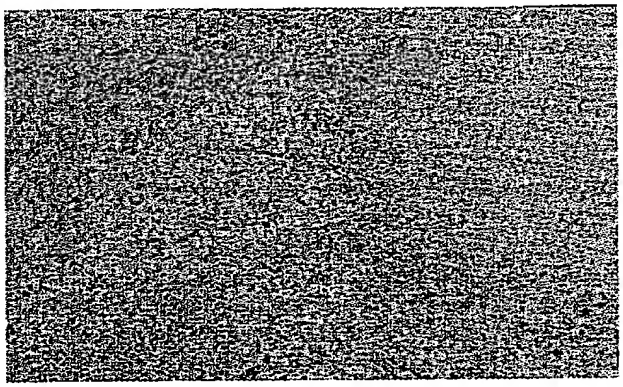
(b)

Fig. 8. Visualisation of apoptotic cells in C6 xenografts after treatment with TPL (magnification 100×: (a) tumour from control mice; (b) tumour from TPL 0.375 g/ml+i.p. (nitroxide 100 mg/kg twice daily, 5 days/week) treated mice.

Table 3
Semi-quantitative evaluation of apoptosis in C6 xenografts excised from control mice and mice treated with TPL according to two different schedules

	No. of preparations	Mean score	% Preparations scoring ⩾3
Control	9	1.9	22
TPL (schedule A)[a]	10	3.25[b]	100
TPL (schedule B)[a]	10	3.5[b]	100

[a] See text for a description of the two treatment schedules.
[b] $P<0.05$ versus control.

microvessel density. However, early angiogenic stages were clearly visible throughout the treated tumour section, indicating that TPL does not cause a complete block of tumour vascularisation, but rather significantly slows down the rate of the process.

Fig. 8 shows the extent of apoptotic damage in representative control (a) and treated preparations (b; high-dose schedule). Semi-quantitative analysis of the data indicated an increase in the incidence of apoptotic cells in tumours from TPL-treated mice at the end of the two weeks of treatment compared with tumours from the untreated animals, even though apoptotic damage was also present to some extent in the control preparations (Table 3).

5. Discussion

Most glioblastomas and anaplastic gliomas are not manageable by current available therapeutic options,

and most patients die within 12 months of diagnosis, independent of treatment modality. A number of reasons may account for the poor response of malignant gliomas to therapy: first, complete surgical removal is practically impossible to achieve, due to active infiltration of the surrounding normal brain tissue by tumour cells. Second, although radiotherapy has been proven to prolong the survival of patients with malignant gliomas to some extent, the development of hypoxic regions within the tumour mass dramatically reduces the efficacy of this approach; third, these tumours respond poorly to most current chemotherapeutic agents, but it is still unclear whether resistance to chemotherapy is intrinsic to glioma cells or due to the presence of the blood–brain barrier, which prevents many drugs from achieving therapeutically relevant concentrations within the tumour [11]. Identification of novel therapeutic agents to inhibit the growth of malignant gliomas and/or to improve their response to chemotherapy is therefore an area of active research.

The results of the present study suggest that the piperidine nitroxide radical TPL may have a role in the pharmacological therapy of gliomas. TPL is a water-soluble, membrane-permeable, low-molecular weight antioxidant which has been shown to protect many biological systems from diverse oxidative insults [6,12,13]. However, evidence has accumulated indicating that nitroxides may also exhibit dose- and time-dependent toxicity in a number of model systems [3,14,15]; this effect has been attributed to the ability of nitroxides to turn from antioxidant to pro-oxidant, depending on the prevailing conditions, and to induce oxidative stress [16]. Previous studies by our group showed that TPL exerts antiproliferative effects on a panel of rodent and human cell lines, with some degree of selectivity between neoplastic and non-neoplastic cells of similar lineage [3]. In the present study, TPL was also found to inhibit the growth of C6 rat glioblastoma cells in a concentration- and time-dependent fashion. Continuous exposure to submillimolar doses of the nitroxide for 96 h was required to effectively inhibit cell proliferation. Interestingly, ST14A cells, a non-tumorigenic cell line derived from rat striatum, are significantly more resistant (approximately 25-fold, data not shown) to TPL than C6 cells, suggesting that some degree of selectivity may also be achieved between neoplastic and non-neoplastic cells derived from the CNS.

Cell death induced by TPL in haematological tumour cells was shown to depend largely upon its ability to activate an apoptotic programme [4], and this paradigm seems to hold for C6 cells as well, at least for short-term exposures. Comparison of the results obtained following incubation with propidium iodide and the TUNEL reaction suggests that cell death occurs mainly by apoptosis at early times (24 h), while later on necrosis may become increasingly important (as shown by the high numbers of cell and nuclear debris at 72 h) with apoptotic levels remaining stable. TPL-induced cell death is associated with increased levels of the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ [4]. Upregulation of $p21^{waf1/cip1}$ often occurs following activation of the *TP53* tumour suppressor gene, but can also be induced by appropriate stimuli in cells lacking a functional p53 protein [4,17,18]. The role played by $p21^{waf1/cip1}$ in the survival/death decision in glioma cells is still a matter of debate. In some instances, overexpression of $p21^{waf1/cip1}$ has been found to enhance clonogenic survival and to suppress apoptosis in brain tumour cell lines, irrespective of the *TP53* status [19]. On the other hand, $p21^{waf1/cip1}$ has been reported to increase glioma cell susceptibility to cisplatin [20], and to suppress tumour growth while increasing radiosensitivity in a rat glioblastoma model [21]. Our results in C6 cells are in line with previous observations: nuclear staining with propidium iodide revealed complete nuclear disaggregation in detached C6 cells following exposure to TPL, suggesting a rapid and dramatic apoptotic process, which was accompanied by concentration-dependent increases in $p21^{waf1/cip1}$. C6 cells have been reported to harbour the wild-type form of the *TP53* gene [22]; however, p53 is expressed at very low levels [23]. In our hands, p53 protein was undetectable under control conditions and unaffected by TPL treatment, suggesting that the $p21^{waf1/cip1}$ increase occurs via p53-independent mechanism(s). Thus, the ability of TPL to induce cell death by a mechanism involving a p53-independent increase in $p21^{waf1/cip1}$ expression could be exploited for the clinical management of gliomas and other tumours lacking a functional p53 protein.

A significant, dose-dependent inhibition of tumour growth was observed when TPL was administered to nude mice bearing C6 xenografts; however, this effect was less potent *in vivo* than expected from *in vitro* cytotoxicity data. As TPL has a very short half-life in plasma [24], long-term exposure, which seems to be required to effectively inhibit C6 cell growth (see above), was achieved by s.c. insertion of an osmotic pump releasing constant amounts of the nitroxide during a 14-day period. Preliminary experiments showed that the 375 mg/ml concentration used for the high-dose treatment group was the highest achievable TPL concentration; when this limit was exceeded, TPL precipitation occurred, impairing drug flow from the pump, besides affecting actual drug concentrations within the pump. To increase TPL systemic levels, albeit transiently, mice were also injected i.p. twice daily with the nitroxide; even so, plasma levels of TPL and related metabolites measured at the end of the treatment period (approximately 15 and 50 μM for the low- and high-dose treatments, respectively; data not shown) were somewhat lower than the concentration required for half-maximal inhibition of C6 growth *in*

vitro, and possibly too low to achieve complete arrest of tumour growth. Thus, *in vivo* results indicate that TPL exerts a significant antitumour effect in the C6 xenograft model; however optimisation of nitroxide delivery may be required to allow full exploitation of the therapeutic potential of TPL as a single agent. On the other hand, a number of observations suggest a possible benefit from the combined use of TPL with other cytotoxic agents: (1) in nude mice, TPL was devoid of general and organ-directed toxic side-effects, and TPL has actually been shown to decrease anthracycline-induced toxic side-effects [12]; (2) a decrease in the rate of tumour vascularisation was observed in TPL-treated animals, suggesting a possible interference of the nitroxide with the angiogenic process. The development of a vascular network is considered to be a rate-limiting step for tumour growth, invasion and metastasis [25], and human glioblastomas usually exhibit a high degree of vascularisation, even though hypoxic areas are fairly common as the tumour mass increases; therefore, inhibition of angiogenesis represents a viable option in glioblastoma treatment [11]. The antiangiogenic effect observed for TPL, possibly mediated through $p21^{waf1/cip1}$ induction in vascular endothelial cells [26], could help restrain tumour growth and facilitate the action of other cytotoxic agents. Further investigations are needed to identify TPL-based combinations which may afford some therapeutic benefit in the treatment of gliomas; preliminary observations by our group indicate that, in both rat and human glioma cell lines, TPL synergises with Temozolomide, an imidazotetrazinone methylating agent with clinical efficacy against malignant gliomas [27,28]. Thus, TPL appears to offer some promise as an adjuvant to conventional chemotherapeutic agents in the clinical management of this highly malignant disease, without unduly adding to the burden of toxic side-effects that generally accompanies such therapeutic regimens.

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